Muscle-targeted hydrodynamic gene introduction of insulin-like growth factor-1 using polyplex nanomicelle to treat peripheral nerve injury

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Abstract

The recovery of neurologic function after peripheral nerve injury often remains incomplete because of the prolonged reinnervation process, which leads to skeletal muscle atrophy and articular contracture from disuse over time. To rescue the skeletal muscle and promote functional recovery, insulin-like growth factor-1 (IGF-1), a potent myogenic factor, was introduced into the muscle by hydrodynamic injection of IGF-1-expressing plasmid DNA using a biocompatible nonviral gene carrier, a polyplex nanomicelle. In a mouse model of sciatic nerve injury, the introduction of IGF-1 into the skeletal muscle of the paralyzed limb effectively alleviated a decrease in muscle weight compared with that in untreated control mice. Histologic analysis of the muscle revealed the IGF-1-expressing plasmid DNA (pDNA) to have a myogenic effect, inducing muscle hypertrophy with the upregulation of the myogenic regulatory factors, myogenin and MyoD. The evaluation of motor function by walking track analysis revealed that the group that received the hydrodynamic injection of IGF-1-expressing pDNA using the polyplex nanomicelle had significantly early recovery of motor function compared with groups receiving negative control pDNA and untreated controls. Early recovery of sensation in the distal area of sciatic nerve injury was also induced by the introduction of IGF-1-expressing pDNA, presumably because of the effect of secreted IGF-1 protein in the vicinity of the injured sciatic nerve exerting a synergistic effect with muscle hypertrophy, inducing a more favorable prognosis. This approach of introducing IGF-1 into skeletal muscle is promising for the treatment of peripheral nerve injury by promoting early motor function recovery.

Keywords

peripheral nerve injury, muscle atrophy, insulin-like growth factor-1 (IGF-1), gene delivery, hydrodynamic injection, polyplex nanomicelle

1. Introduction

The recovery of neurologic function after peripheral nerve injury is often incomplete because of the prolonged reinnervation process. A variety of approaches directed at enhancing nerve regeneration has been investigated using nerve growth factors such as fibroblast growth factor-2 (FGF-2) [1], glial cell line-derived neurotrophic factor (GDNF) [2, 3], brain-derived neurotrophic factor (BDNF) [4], and vascular endothelial growth factor (VEGF) [5, 6]. However, the inherently limited rate of axonal regrowth leads to muscle atrophy and articular contracture because of disuse over time, hampering the full recovery of motor function despite physical therapy and additional treatments.

Insulin-like growth factor-1 (IGF-1) is a potent myogenic factor. Several basic studies using transgenic mice overexpressing IGF-1 have revealed that IGF-1 promotes muscle regeneration by regulating myoblast proliferation, myogenic differentiation, and myotube hypertrophy [7-10]. Thus, administration of IGF-1 has potential as a treatment to rescue muscles affected by disuse atrophy, thereby promoting functional recovery after injury.

Therefore, the introduction of a gene expressing IGF-1 is believed to be an effective treatment strategy. Although several approaches using IGF-1 recombinant protein or its fusion either by systemic injection or intramuscular injection showed efficacy for the induction of muscle hypertrophy [11-13], the use of gene introduction has advantages in providing higher and sustained IGF-1 expression in skeletal muscle [14]. Various trials of IGF-1 gene administration using naked plasmid DNA (pDNA) with electroporation or an adeno-associated virus vector showed an effect on inducing muscle hypertrophy in animal models of muscle disuse or myotoxic injury [15-19]. However, with regard to clinical availability, there still are problems associated with gene introduction, such as the highly invasive manner of electroporation and risks of natural viruses. Moreover, although the effect of IGF-1 on muscle fibers has been intensely studied by histologic and molecular biologic analyses, the final outcome on the recovery of motor function remains unclear.

In this study, we proposed a novel approach to introduce IGF-1-expressing

pDNA with high efficacy and minimal tissue damage to the muscle, hydrodynamic injection of polyplex nanomicelle into the skeletal muscle. Hydrodynamic injection is a promising approach to enhance transgene expression due to the transient increase in hydrostatic pressure in the target tissue and organ [20, 21]. For targeting skeletal muscle, we previously reported that our original nonviral gene carrier, polyplex nanomicelle, effectively provided high transgene expression following hydrodynamic injection into a distal vein with isolation of the limb by tourniquet [22, 23].

The polyplex nanomicelle is a nonviral carrier composed of pDNA and poly(ethyleneglycol) (PEG)-block-polycation, forming a micelle structure with a diameter of several-tens nm surrounded by a hydrophilic and electrically neutral palisade of PEG [23, 24]. This structure increases the steric stability of polyplexes under physiological conditions and exhibits less nonspecific interaction with biologic components such as serum proteins. In addition, our original polycation, poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PAsp(DET)], used as a core-forming segment of polyplex nanomicelles, was developed to increase transfection efficiency with minimal cytotoxicity due to its self-catalytic degradability under physiological conditions [25, 26]. Using this system, we have already achieved therapeutic effects including bone regeneration in critical bony defects of mice by introducing osteogenic transcription factors [27] and regulation of right heart pressure in a mouse model of idiopathic pulmonary hypertension by introducing adrenomedullin [28].

Based on the properties described, the nanomicelle is beneficial to increase cellular uptake and subsequent high transgene expression in skeletal muscle compared with the hydrodynamic injection of naked pDNA [22]. Moreover, the nanomicelle allows uniformly-distributed transgene expression in the muscle fibers, presumably due to the well-regulated particle size of several-tens nm that improves tissue penetration upon injection. In addition, the combined use of an anionic polysaccharide, chondroitin sulfate (CS), significantly reduced the tissue damage and inflammatory responses in skeletal muscle after the hydrodynamic injection, leading to prolonged transgene expression for more than a month [29].

We applied this method to introduce IGF-1-expressing pDNA into the skeletal

muscle to treat mice with sciatic nerve injury. The effect of IGF-1 on muscle hypertrophy and the resulting therapeutic effect on motor function were thoroughly investigated, including a behavioral analysis of mice. Based on the outcomes, the applicability of this system for the treatment of peripheral nerve injury is discussed in detail.

2. Materials and Methods

2.1 Materials

Plasmid DNAs (pDNAs) encoding IGF-1 and β -galactosidase (β -gal) were purchased from InvivoGen (pBLAST44-mIGF-1a; San Diego, CA, USA) and Promega (pSV- β -galactosidase; Madison, WI, USA), respectively. The pDNA concentration was determined by reading the absorbance at 260 nm. Chondroitin sulfate A (CS) and streptozotocin were purchased from Sigma Aldrich (St Louis, MO, USA).

2.2 Animals

Balb/c albino mice (female; 10–14 weeks old) were purchased from Charles River Laboratories (Tokyo, Japan). All animal experimental protocols were performed in accordance with the approval of the Animal Care and Use Committee of the University of Tokyo.

2.3 Preparation of polyplex nanomicelles in combination with CS

For hydrodynamic introduction of pDNA into skeletal muscle, a system of polyplex nanomicelles based on a block copolymer of PEG and poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} [PEG-PAsp(DET)] was used. This block copolymer was synthesized as previously reported [25]. The molecular weight of PEG was 12,000, and the polymerization degree of the PAsp(DET) segment was determined to be 69 by $^1\text{H-NMR}$. The block copolymer and pDNA were separately dissolved in 10 mM Tris-HCl buffer (pH 7.4). PEG-PAsp(DET)/pDNA polyplex nanomicelles were obtained by simply mixing both solutions.

Before hydrodynamic injection, CS solution was added to the nanomicelle solution because we had observed that CS had remarkable effects on a reduction in tissue damage and prolonged transgene expression after hydrodynamic injection into skeletal muscle [29]. Based on the study, the mixing ratio of amino groups in PEG-PAsp(DET), phosphate groups in pDNA, and the total carboxyl and sulfate groups in CS was set to 20:1:100 as the optimal condition to obtain efficient transgene expression. The final concentration of pDNA was adjusted to 134 $\mu\text{g/mL}$. Immediately

before injection into mice, a 1/100 volume of 5M NaCl solution was added to form a half-isotonic solution.

2.4 Hydrodynamic injection into the limb vein of mice

Hydrodynamic gene introduction into skeletal muscle was performed as previously reported [22]. In brief, after anesthetizing mice with 3% isoflurane (Abbott Japan Co., Ltd., Tokyo, Japan), a tourniquet was placed on the proximal thigh to transiently restrict blood flow. From a distal site of the great saphenous vein, the nanomicelle solution or naked pDNA (375 μ L; containing 50 μ g pDNA) was injected over 5 s. At 5 min after the injection, the tourniquet was released. For negative controls, the same volume of physiological saline was injected using the same procedure.

2.5 Intramuscular injection into the limb

The nanomicelle solution or naked pDNA was prepared similarly as the case for hydrodynamic injection at greater concentration of pDNA (50 μ L; containing 50 μ g pDNA). The injection was done for the center of muscle belly of quadriceps femoris.

2.6 Surgical procedure to create sciatic nerve injury

One hour after the hydrodynamic injection, a surgical procedure was performed to create a cryo-injury of the sciatic nerve [30]. A skin incision was made at the right greater trochanter, and the right sciatic nerve was carefully exposed without damaging the muscle. At a point immediately distal to the gluteus maximus muscle, the nerve was grasped for 30 s using hemostatic forceps, which had been chilled in advance with liquid nitrogen for 30 s. This procedure was performed twice.

2.7 Real-time polymerase chain reaction (PCR)

To evaluate the IGF-1, MyoD and myogenin gene expression levels in the muscle, the mRNA expression levels were evaluated using real-time PCR. Seven days after the hydrodynamic injection, the mice were euthanized and the muscle of quadriceps femoris and triceps surae resected. The mRNA were purified from the muscle samples using RNeasy Fibrous Tissue Mini Kits (50) (Qiagen, Hilden, Germany).

After reverse transcription using a QuantiTect Reverse Transcription Kit (Qiagen), real-time PCR was performed with QuantiTect SYBR Green PCR Master Mix (Qiagen) using an ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA). All expression data were normalized to the mRNA levels of β -actin. The primer sequences used were: IGF-1, forward primer TGGATGCTCTTCAGTTCGTG, reverse primer GTCTTGGGCATGTCACTGTG; β -actin, forward primer AGATGTGGATCAGCAAGCAG, reverse primer GCGCAAGTTAGGTTTTGTTCA. For myoD and myogenin, Taqman® gene expression assays were used (MyoD: Mm01203489_g1, myogenin: Mm00446195_g1). The $\Delta\Delta C_t$ method was used to calculate the relative expression ratio.

2.8 Evaluation of muscle atrophy

The muscle weight was evaluated 7 days after injection by resecting the whole triceps surae mass from its origin at the popliteal fossa to insertion at the Achilles' tendon.

For histological analyses of the muscle to evaluate the muscle fiber hypertrophy, we obtained semi-thin fresh-frozen sections 7 or 16 days after the injection by a method using adhesive film (Cryofilm type IIC9, Section-lab, Hiroshima, Japan) [31, 32]. This method allows for the preparation of sections from tissue samples containing calcified bone. In brief, mice were euthanized, and the limbs were resected and frozen in cooled isopentane. The frozen limbs were subsequently embedded in SCEM compound (Section-lab, Hiroshima, Japan). After the cut surface was covered with an adhesive film, frozen sections (for quadriceps femoris: 10- μ m thick, 10-mm proximal from upper edge of patella, for triceps surae: 10- μ m thick, 10-mm proximal from the heel) were obtained using a CM 3050 S cryostat (Leica Microsystems, Wetzlar, Germany). The sections were stained with hematoxylin and eosin, and cross-sectional areas of individual muscle fibers were analyzed using image analysis software, ImageJ.

2.9 Walking track analysis

For the evaluation of motor function recovery, walking track analysis was performed [33]. Paw prints of the mice were recorded by moistening the hind paws of

each animal with black ink and having them walk a 4.5 × 35-cm corridor in a darkroom. The tracks were evaluated for two different parameters: toe spread (TS), the distance between the first and fifth toes, and print length (PL), the distance between the third toe and the hind pad. The sciatic functional index (SFI) was calculated using the following formula:

$$\text{SFI} = 118.9 \times (\text{ETS} - \text{NTS})/\text{NTS} - 51.2 \times (\text{EPL} - \text{NPL})/\text{NPL} - 7.5$$

where EPL and ETS were the experimental print length and experimental toe spread, respectively, taken from the right paw, which received the sciatic nerve injury, and NPL and NTS were the normal print length and normal toe spread, respectively, taken from the normal left paw. In a preliminary experiment, normal mice without sciatic nerve injury had SFI values on the average of -12.4 ± 4.8 ($n = 4$); therefore, we set the normal range as >-15 in this experiment.

2.10 Preparation of mice diabetic model

To induce diabetes, a single intraperitoneal injection of streptozotocin (200 mg/kg body weight) was administrated to the mice [34]. Blood glucose levels were measured using Fuji DRI-CHEM slide GLU-WIII and DRI-CHEM 7000i (Fujifilm, Tokyo, Japan) seven days after the injection of streptozotocin. The mice exhibiting blood glucose levels >300 mg/dL served as diabetic animals for subsequent experiments.

2.11 Analyses of sensation

To evaluate the sensation in the paw after sciatic nerve injury, we performed two individual tests following procedures previously established. Constant pressure was applied to the soles of the feet with a clip. Paw withdrawal latencies were measured as an indication of sensitivity to pain [35]. To avoid overstimulation, each experiment was limited to 30 s.

In addition, we analyzed the response to cold [36]. After dipping the impaired hind paw into acetone, mice were immediately moved to a plastic cage, and the number of brisk foot withdrawal responses to the sensation of cold induced by

evaporation of the acetone was measured.

2.12 Statistical analysis

Statistical analyses were done using one-way factorial analysis of variance (ANOVA) followed by the *post hoc* Tukey-Kramer correction for multiple comparisons. In all statistical analyses, significance was defined as $P < 0.05$.

3. Results

3.1. Effect of IGF-1-expressing pDNA to alleviate muscle atrophy after sciatic nerve injury

The IGF-1 expression levels in the quadriceps femoris and triceps surae were evaluated seven days after hydrodynamic injection of IGF-1-expressing pDNA using real-time PCR. The surgical treatment to induce sciatic nerve injury was performed to coincide with the injection of pDNA. In mice that received polyplex nanomicelles, the expression level of IGF-1 in the muscle was 37 times higher in quadriceps femoris and four times higher in triceps surae than those in untreated control mice (Fig. 1A, C). Injection of the identical amount of naked pDNA also showed an increase in IGF-1 expression; however, the level was lower than that identified in mice that received the nanomicelle. The myogenic regulatory factors myogenin and MyoD, known to be key molecules regulating muscle differentiation, showed upregulation in response to the introduction of IGF-1-expressing pDNA compared with saline controls and untreated controls. For quadriceps femoris, the group receiving nanomicelle induced significantly greater upregulation of myogenin and MyoD compared with naked pDNA, although there were no difference between the two groups receiving pDNA by nanomicelle or naked pDNA for triceps surae (Fig. 1B, D).

Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1 Gene expression in skeletal muscle of quadriceps femoris (A, B) and triceps surae (C, D) after hydrodynamic injection, evaluated by real-time polymerase chain reaction (PCR) to determine mRNA expression levels of IGF-1 (A, C) and myogenic regulatory factors (myogenin and MyoD) (B, D). Data are presented as relative values to the uninjected control, as means $\pm$ standard error of the mean (SEM; $n = 4$ for quadriceps femoris and $n=5$ for triceps surae), $*P < 0.05$ vs. saline; $\dagger P < 0.05$ vs. uninjected control; $\ddagger p < 0.05$ vs. IGF-1 (naked pDNA).

To evaluate the effects of IGF-1-expressing pDNA as a potent myogenic factor, the whole muscle weight was examined after resection of bilateral triceps surae seven days after sciatic nerve injury and concomitant hydrodynamic injection to the right leg. Compared with the normal opposite side (left) leg, the muscle weight of the right leg decreased to 65% in mice that received a hydrodynamic injection of saline and untreated control mice. The decrease for the mice receiving a hydrodynamic injection of naked IGF-1-expressing pDNA was alleviated to 68% to the normal leg, and to 81% for the mice that received a hydrodynamic injection of polyplex nanomicelle containing pDNA (Fig. 2). It is noted that the weight of the normal left leg showed small difference (~ 10%) among the four groups, however, there were no significant difference among the values. Although the reason for the difference in the muscle weight of the left leg remains unclear, it is confirmed that IGF-1-expressing pDNA showed a therapeutic effect to alleviate muscle weight decrease of the right leg induced by sciatic nerve injury. (Fig. 2).

Fig. 2

Fig. 2 Triceps surae muscle weight after right leg sciatic nerve injury, followed by the hydrodynamic injection of IGF-1-expressing pDNA or saline. Observed muscle weight values of right (injured) and left (normal) legs were presented. Data are expressed as means $\pm$ SEM ($n = 5$), * $P < 0.05$ vs. right leg of uninjected control mice; † $P < 0.05$ vs. right leg of saline.

3.2. IGF-1-expressing pDNA induced hypertrophy of muscle fibers

To directly evaluate the effect of IGF-1 on muscle fibers, we performed histologic analysis of the muscle after hydrodynamic injection of IGF-1-expressing pDNA. Cross-sections of quadriceps femoris were obtained 7 days after the injection to determine the average cross-sectional areas of individual muscle fibers. As shown in Fig. 3AB, the group receiving nanomicelle showed significantly larger area of muscle fibers compared with other groups of receiving IGF-1-expressing pDNA (naked pDNA), β -gal-expressing pDNA (nanomicelle or naked pDNA), and saline. Thus, it is suggested that the nanomicelle effectively introduced IGF-1 into the muscle to induce muscle hypertrophy through the upregulation of myogenic regulatory factors. The long-term effect of IGF-1 was also evaluated for triceps surae using nanomicelle. Although the muscle fiber area showed time-dependent decrease from Day 7 to Day 16 due to disuse atrophy, IGF-1-expressing pDNA introduced by nanomicelle showed a sustained effect to alleviate the atrophy to a significantly greater extent compared with saline control (Supplemental Fig. 1AB). There were no apparent differences in the histopathology findings with regard to the muscle fibers (Fig. 3A and Supplemental Fig. 1A).

Fig. 3 Histologic analyses of muscle after hydrodynamic injection of IGF-1-expressing pDNA , β-galactosidase-expressing pDNA (using nanomicelle or by the form of naked pDNA) or saline. (A) Histology sections of quadriceps femoris on day 7 after sciatic nerve injury. (scale bars, 50 μm). (B) Cross-sectional areas of individual muscle fibers in quadriceps femoris. More than 100 muscle fibers were subjected for statistical analyses. Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β-gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); ‡ $P < 0.05$ vs. β-gal (nanomicelle).

3.3. IGF-1-expressing pDNA promoted early recovery of motor function

To evaluate the effect on motor function, we evaluated walking track data. The sciatic functional index (SFI) was calculated from the footprints (see Materials and Methods). Up to day 10 following sciatic nerve injury, all mice including those receiving IGF-1-expressing pDNA, negative control pDNA (expressing β -galactosidase), and untreated control mice showed almost no recovery of motor function (Fig. 4A). However, after that time, the group that received the hydrodynamic injection of IGF-1-expressing pDNA using polyplex nanomicelle showed significantly early recovery of motor function compared with other groups. The results are depicted in Figure 4B, where the numbers of mice showing full recovery represented by a SFI value >-15 are presented. By day 18, all mice that received the hydrodynamic injection of IGF-1-expressing pDNA using polyplex nanomicelle achieved full recovery, whereas in the other groups, not all mice reached full recovery even at day 25.

To compare the injection method of hydrodynamic injection with direct intramuscular (i.m.) injection, we performed the i.m. injection of IGF-1-expressing pDNA using nanomicelle or by the form of naked pDNA. As shown in Fig. 4C, the group of i.m. injection (nanomicelle and naked pDNA) showed similar profiles of motor function recovery as the control uninjected group, whereas the group receiving hydrodynamic injection showed significantly more rapid recovery. Thus, it is strongly suggested that the hydrodynamic injection is likely to be advantageous to achieve the therapeutic effect with the combined use of nanomicelle.

Fig. 4A

Fig. 4B

Fig. 4C

Fig. 4 Walking track analyses after sciatic nerve injury. (A) Sciatic functional index (SFI) calculated from footprints after hydrodynamic injection of IGF-1-expressing pDNA or β -galactosidase-expressing pDNA (using nanomicelle or by the form of naked pDNA). Data are expressed as means ($n = 6$). Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β -gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); & $P < 0.05$ vs. β -gal (nanomicelle). (B) Numbers of mice showing recovery of motor function to SFI values > -15 in the experiment of (A). (C) Sciatic functional index (SFI) after intramuscular (i.m.) injection of IGF-1-expressing pDNA (using nanomicelle or by the form of naked pDNA) or hydrodynamic (hydro.) injection of nanomicelle. Data are expressed as means ($n = 3$). Error bars are SEM, * $P < 0.05$ vs. uninjected control; † $P < 0.05$ vs. i.m. of naked pDNA; ‡ $P < 0.05$ vs. i.m. of nanomicelle.

To further investigate the applicability of this treatment approach for more intractable conditions, we used a mouse model of diabetes mellitus. It is well known that peripheral nerve regeneration tends to be markedly impaired in association with diabetes mellitus, causing neuropathy with a poor prognosis [37, 38]. Similar to that described for normal mice, the sciatic nerve injury and concomitant hydrodynamic injection were applied to diabetic mice, and motor function was evaluated by walking track analysis. As shown in Figure 5, the introduction of IGF-1-expressing pDNA using the polyplex nanomicelle promoted early recovery of SFI values compared with other groups similar to the results found with normal mice.

Fig. 5 Walking track analyses after sciatic nerve injury using diabetes mellitus disease model mice. Analyses were performed similar to those for wild-type mice (as presented in Fig. 4). Data are expressed as means ($n = 5$). * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β-gal (naked pDNA); ‡ $P < 0.05$ vs. β-gal (nanomicelle).

3.4. IGF-1 also promoted early recovery of sensation after sciatic nerve injury

Using the disease mouse model of diabetes mellitus, sensation in the distal area of sciatic nerve injury was evaluated after introduction of IGF-1-expressing pDNA. The paw pressure and acetone test were used to evaluate sensory changes (see Materials and Methods). In the paw pressure test used to determine sensation to

mechanical compression, the mice that received IGF-1-expressing pDNA using the polyplex nanomicelle showed remarkably shorter latencies to mechanical compression even on day 2 after sciatic nerve injury compared with other groups that received the naked form of IGF-1-expressing pDNA, negative control pDNA (expressing β -galactosidase), or untreated control mice (Fig. 6). In accordance with the results of motor function recovery, the control groups exhibited recovery to the normal level sensation around day 16.

The acetone test used to evaluate sensation to cold also revealed early recovery with introduction of IGF-1-expressing pDNA using polyplex nanomicelle, although the differences among the groups were not as prominent as that experienced with the paw pressure test (Fig. 7). The control groups showed recovery to the normal level by day 22.

Fig. 6

Fig. 6 Analyses of pain sensation in the paw after sciatic nerve injury. Data are expressed as means ($n = 5$). Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β-gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); ‡ $P < 0.05$ vs. β-gal (nanomicelle).

Fig. 7

Fig. 7 Analyses of cold sensation after sciatic nerve injury. Data are expressed as means ($n = 5$). Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β-gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); ‡ $P < 0.05$ vs. β-gal (nanomicelle).

4. Discussion

In this study, the efficacy of hydrodynamic introduction of IGF-1-expressing pDNA into skeletal muscle was demonstrated for the treatment of peripheral nerve injury. The expected role of IGF-1 is based on its myogenic functions to alleviate muscle atrophy gradually induced during a period of denervation. Although numerous approaches have been attempted to enhance nerve regeneration at the injury site, the inherently limited rate of axon elongation requires a prolonged period of treatment and leads to severe muscle atrophy and subsequent irreversible contracture of muscle and joint. The unique point of this study involves the direct targeting of the paralyzed muscle for treatment of peripheral nerve injury.

Compared with the routine method of intramuscular injection, which tends to induce severe tissue damage at the injected site, the hydrodynamic injection of polyplex nanomicelle into skeletal muscle is highly suitable to alleviate atrophy for the following reasons: 1) damage to the muscle is minimal and reversible and 2) this technique allows uniform distribution of gene introduction in the muscle fibers [22], which is apparently advantageous to obtain overall hypertrophy in the muscle fibers and consequent muscle-strengthening effects. With regard to the safety, the hydrodynamic mechanism inevitably causes a temporary elevation of creatine phosphokinase (CPK) in the serum due to transient enhancement of the permeability of the plasma membrane; however, the CPK value returns to normal in a few days without causing any symptoms [21, 22]. In addition, the nanomicelle was formed with the addition of anionic CS, which effectively reduced the inflammatory responses that may be induced by cationic polymers, leading to sustained transgene expression in the muscle [29]. Based on these features, the hydrodynamic injection of IGF-1-expressing pDNA using nanomicelle achieved the early recovery of motor function not only in wild-type mice (Fig. 4) but also in those with diabetes mellitus (Fig. 5).

Furthermore, the hydrodynamic injection is suitable for the treatment of peripheral nerve injury because this technique allows extensive pDNA introduction into muscles distal to the tourniquet. Paralysis occurs in all muscles distal to the nerve injury; thus, treatment should target all of these affected muscles. Although it is

difficult to directly evaluate the individual functions of small muscles such as intrinsic muscles in the mouse foot, the effects on the small muscles were partially represented by SFI values, which contained the parameter of intrinsic muscle strength as represented by toe spread. It is reasonable to assume that the capability of efficient pDNA introduction into the distal small muscles by hydrodynamic injection may contribute to the early recovery of motor functions after nerve injury.

It is interesting that, although the high transgene expression of IGF-1 was similarly obtained for quadriceps femoris and triceps surae after hydrodynamic injection of nanomicelle compared with that of naked pDNA, the effects on upregulating the myogenic regulatory factors (myogenin and myoD) were particularly more prominent in quadriceps femoris compared with triceps surae (Fig. 1B and D). A possible reason is that the femur lesion is more distant from the injection site (great saphenous vein), and the tissue damage and subsequent inflammatory responses are relatively lower than those in the lower leg, resulting in the clearer effects of IGF-1 introduction to induce muscle hypertrophy in quadriceps femoris.

Surprisingly, early recovery of sensation was also induced by the introduction of IGF-1-expressing pDNA. Although some papers reported that IGF-1 introduction promoted sensation recovery [39-41], the effects of IGF-1 on the sensory nerves were not as clear as those regarding the myogenic effect. It is probable that a significant amount of IGF-1 protein was secreted from the muscle, similar to that discussed in our previous paper, where the introduction of pDNA expressing a soluble form of vascular endothelial growth factor (VEGF) receptor-1 (sFlt-1) showed a remarkable effect on tumor growth regression by the secretion of sFlt-1 protein into the blood from the muscle [22]. Similar to sFlt-1, IGF-1 protein is likely to be secreted in the vicinity of the sciatic nerve injury site, promoting early recovery of sensation. Another possibility is that the muscle hypertrophy induced by IGF-1 caused an afferent effect on sensory nerves. Such effects are reported in physical training. For example, treadmill training stimulates the distal portion of the injured nerve and promotes nerve regeneration through activation of Schwann cell proliferation [42]. The detailed mechanism involved in rapid sensation recovery remains an unsolved issue; however, it is clear that rapid sensation recovery exerts a synergistic effect with muscle hypertrophy to produce a

more favorable prognosis.

In conclusion, the introduction of IGF-1-expressing pDNA into skeletal muscle provided a remarkable therapeutic effect, promoting the recovery of motor function in a mouse model of sciatic nerve injury. The hydrodynamic injection of polyplex nanomicelle containing IGF-1-expressing pDNA maximized the effect of IGF-1 without causing any apparent tissue damage. This treatment achieved remarkable effects by alleviating disuse muscle atrophy and promoting early recovery of motor function. We consider this approach to have potential to be used as the treatment for peripheral nerve injury.

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Figure legends

Fig. 1

Gene expression in skeletal muscle of quadriceps femoris (A, B) and triceps surae (C, D) after hydrodynamic injection, evaluated by real-time polymerase chain reaction (PCR) to determine mRNA expression levels of IGF-1 (A, C) and myogenic regulatory factors (myogenin and MyoD) (B, D). Data are presented as relative values to the uninjected control, as means $\pm$ standard error of the mean (SEM; $n = 4$ for quadriceps femoris and $n=5$ for triceps surae), $*P < 0.05$ vs. saline; $\dagger P < 0.05$ vs. uninjected control; $\ddagger p < 0.05$ vs. IGF-1 (naked pDNA).

Fig. 2

Triceps surae muscle weight after right leg sciatic nerve injury, followed by the hydrodynamic injection of IGF-1-expressing pDNA or saline. Observed muscle weight values of right (injured) and left (normal) legs were presented. Data are expressed as means $\pm$ SEM ($n = 5$), $*P < 0.05$ vs. right leg of uninjected control mice; $\dagger P < 0.05$ vs. right leg of saline.

Fig. 3

Histologic analyses of muscle after hydrodynamic injection of IGF-1-expressing pDNA, β -galactosidase-expressing pDNA (using nanomicelle or by the form of naked pDNA) or saline. (A) Histology sections of quadriceps femoris on day 7 after sciatic nerve injury. (scale bars, 50 μ m). (B) Cross-sectional areas of individual muscle fibers in quadriceps femoris. More than 100 muscle fibers were subjected for statistical analyses. Error bars are SEM, $*P < 0.05$ vs. saline; $\dagger P < 0.05$ vs. β -gal (naked pDNA); $\# P < 0.05$ vs. IGF (naked pDNA); $\ddagger P < 0.05$ vs. β -gal (nanomicelle).

Fig. 4

Walking track analyses after sciatic nerve injury. (A) Sciatic functional index (SFI) calculated from footprints after hydrodynamic injection of IGF-1-expressing pDNA or β -galactosidase-expressing pDNA (using nanomicelle or by the form of naked pDNA). Data are expressed as means ($n = 6$). Error bars are SEM, $*P < 0.05$ vs. saline; $\dagger P < 0.05$

vs. β -gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); & $P < 0.05$ vs. β -gal (nanomicelle). (B) Numbers of mice showing recovery of motor function to SFI values >-15 in the experiment of (A). (C) Sciatic functional index (SFI) after intramuscular (i.m.) injection of IGF-1-expressing pDNA (using nanomicelle or by the form of naked pDNA) or hydrodynamic (hydro.) injection of nanomicelle. Data are expressed as means ($n = 3$). Error bars are SEM, * $P < 0.05$ vs. uninjected control; † $P < 0.05$ vs. i.m. of naked pDNA; ‡ $P < 0.05$ vs. i.m. of nanomicelle.

Fig. 5

Walking track analyses after sciatic nerve injury using diabetes mellitus disease model mice. Analyses were performed similar to those for wild-type mice (as presented in Fig. 4). Data are expressed as means ($n = 5$). * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β -gal (naked pDNA); ‡ $P < 0.05$ vs. β -gal (nanomicelle).

Fig. 6

Analyses of pain sensation in the paw after sciatic nerve injury. Data are expressed as means ($n = 5$). Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β -gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); ‡ $P < 0.05$ vs. β -gal (nanomicelle).

Fig. 7

Analyses of cold sensation after sciatic nerve injury. Data are expressed as means ($n = 5$). Error bars are SEM, * $P < 0.05$ vs. saline; † $P < 0.05$ vs. β -gal (naked pDNA); # $P < 0.05$ vs. IGF (naked pDNA); ‡ $P < 0.05$ vs. β -gal (nanomicelle).